

Osher EL, Castillo F, Elumalai N, Waring MJ, Pairaudeau G, Tavassoli A.
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Bioorganic & Medicinal Chemistry 2018

DOI: <https://doi.org/10.1016/j.bmc.2018.03.012>

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DOI link to article:

<https://doi.org/10.1016/j.bmc.2018.03.012>

Date deposited:

15/03/2018

Embargo release date:

09 March 2019



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A Genetically Selected Cyclic Peptide Inhibitor of BCL6 Homodimerization

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We report an inhibitor of the homodimeric protein-protein interaction of the BCL6 oncoprotein, identified from a genetically encoded SICLOPPS library of 3.2 million cyclic hexapeptides in combination with a bacterial reverse two-hybrid system. This cyclic peptide is shown to bind the BTB domain of BCL6, disrupts its homodimerization, and subsequent binding of the SMRT2 corepressor peptide.

Introduction

The B-cell lymphoma 6 (BCL6) transcription factor[1, 2] is a master regulator of the germinal centre, which acts by repressing a plethora of critical genes involved in the DNA damage response and cell cycle checkpoints.[3-6] Aberrant expression of BCL6 is associated with the uncontrollable growth and proliferation of B-cells, driving the malignant diffuse large B-cell lymphoma (DLBCL) phenotype. The BCL6 proto-oncogene encodes a 706 amino acid (79 kDa) zinc finger transcription factor, which is comprised of three distinct domains.[2] The N-terminal bric-a-brac, tramtrack and broad-complex (BTB) domain (residues 5-129) plays a fundamental role in the activity of BCL6 by mediating obligate homodimerization and subsequently recruiting co-repressor molecules that repress target genes in multi-protein histone deacetylase complexes.[7] Thus BTB homodimerization is an absolute requirement and critical for BCL6 repressor functions; upon dimerization, each BTB monomer forms a tight intertwined butterfly like homodimer in which approximately a quarter of the monomer surface is masked upon dimer formation.[8] The only prerequisite for the correct folding of the BTB domain of BCL6 is homodimerization, and the interaction is obligate in nature, with an unmeasurable affinity.[9] Dimerization generates a symmetrical binding site at the interface of the two domains known as the lateral groove. This groove creates a docking site that allows specific binding of three co-repressor proteins, nuclear-receptor co-repressor 1 (NCoR1), and silencing mediator for retinoid and thyroid receptor (SMRT) and BCL6-co-repressor (BCoR) to bind exclusively.[10-12] Over the past decade, BTB-SMRT/BCoR heterodimerization has been extensively explored and

a number of inhibitors of this Protein-protein interaction (PPI) have been reported.[13-17] There are currently no BTB inhibitors in the clinic, and there are no molecules reported that inhibit BTB homodimerization. Given our prior successes with a genetically encoded high-throughput screening platform,[18-21] we sought to probe the limits of our methodology by aiming to identify inhibitors of BTB homodimerization using this system.

Results

Constructing the BTB RTHS and SICLOPPS screening

We began by constructing a bacterial reverse two-hybrid system (RTHS),[22] where survival and growth of the host *E. coli* would be dependent on inhibition of BTB homodimerization (Figure 1a). In this RTHS, the targeted protein is expressed as a fusion with the 434 repressor protein; homodimerization of the targeted protein (BTB in this case) results in formation of a functional 434 repressor that binds to engineered chromosomal operators, and prevents reporter-gene expression (Figure 1a). Disruption of the target PPI will also disrupt the 434 repressor complex, leading to reporter gene expression and growth of the host strain on selective media (Figure 1a). Performance of the constructed BTB RTHS was initially assessed by the function of protein encoded by the LacZ reporter gene (β -galactosidase) using an *o*-nitrophenyl- β -D-galactopyranoside (ONPG) assay. In line with repressor formation, an IPTG-dependent reduction in LacZ activity was observed in this assay (Figure 1b). We further assessed the BTB RTHS by ten-fold serial dilution drop-spotting on selective media; formation of a functional repressor would be expected to lead to cell death on selective media (due to the absence of expression of the first and second reporter genes HIS3 and KanR). We observed significant inhibition of growth at 5 μ M IPTG (Figure 1c), confirming the formation of a functional repressor. We next used this BTB RTHS in combination with a 3.2 million member SICLOPPS CX₅ cyclic peptide library (where X = any proteinogenic amino acid); a cyclic peptide inhibitor of BTB homodimerization would also disrupt the 434 repressor, and consequently result in survival of a bacterial host on selective media (Figure 1a). After transformation with the SICLOPPS plasmid library, approximately 10⁷ *E. coli* transformants were plated onto selective media plates supplemented with L-arabinose (inducer of cyclic peptide production). Around 4000 surviving colonies were observed, and these were individually assessed for their ability to disrupt the BTB homodimer using the BTB RTHS.[23] Activity of the selected peptides were ranked by drop spotting, with a single peptide illustrating significantly more potent activity (Figure 1C, bottom row). The identity of this peptide was deconvoluted as *cyclo*-CIYYCV by isolating and sequencing the parent SICLOPPS

plasmid. This cyclic peptide was subsequently synthesized by solid-phase peptide synthesis for use in the following *in vitro* assays.

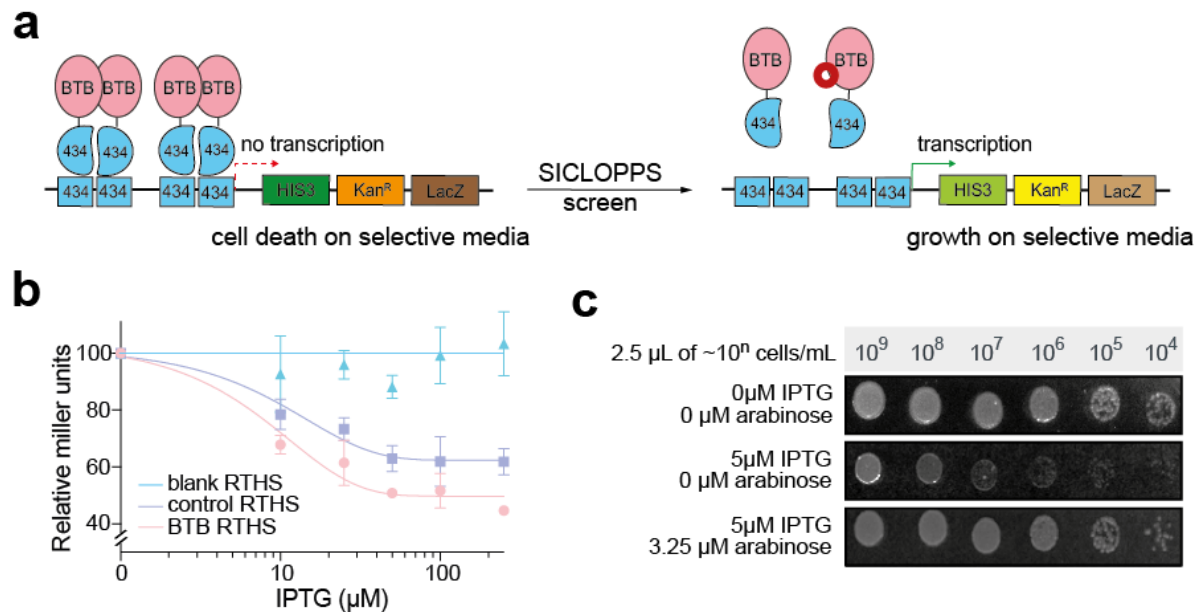


Figure 1: Screening a SICLOPPS library with the BTB RTHS. (a) BTB is expressed as a fusion with the 434 bacteriophage repressor protein. Homodimerization of BTB reconstitutes the repressor, preventing transcription of three reporter genes. An inhibitor of BTB homodimerization will also disrupt repressor formation, enabling expression of the reporter genes, leading to survival and growth of the BTB RTHS on selective media. (b) ONPG assay shows a loss of β -galactosidase activity (LacZ reporter gene product) in response to an increase in IPTG concentrations, indicating formation of a functional repressor. Data presented as Mean \pm SEM, $n=3$. (c) Drop-spotting 10-fold serial dilutions (2.5 μ L of 10^n cells/mL) of the BTB RTHS. In the absence of IPTG and arabinose, full growth is observed, whereas in the presence of 5 μ M IPTG growth of the RTHS is repressed by ~ 4 spots. In the presence of 3.25 μ M arabinose (inducing SICLOPPS) and 5 μ M IPTG growth of the RTHS is restored, due to disruption of the BTB PPI.

Assessing the binding of *cyclo*-CIYYCV to BTB *in vitro*

The ability of *cyclo*-CIYYCV (figure 2a) to bind to the BTB homodimer was probed *in vitro* using two recombinant proteins. The first was a BTB₅₋₁₂₉ wild-type (WT) protein that required a non-cleavable glutathione S-transferase (GST) tag to enhance its solubility. The second variant of the BTB protein was a previously reported triple cysteine mutant C8Q, C67R and C84N (3CysMut from herein); these mutations have been shown to significantly enhance solubility and prevented aggregation when expressed with a His₆ tag.[9, 24, 25] The GST-BTB WT and 3CysMut proteins were used in parallel to characterize the identified inhibitor, to ensure that neither the GST tag, nor the 3 cysteine mutations were significantly altering the native state of the

protein. Given that our inhibitor contains two cysteines, it may also be forming disulfide bonds with the protein. We initially quantified the binding affinity of *cyclo*-CIYYCV to BTB by microscale thermophoresis (MST). We observed an apparent K_D value of $142 \pm 25 \mu\text{M}$ when using GST-BTB (figure 2b) and $50 \pm 11 \mu\text{M}$ when using 3CysMut BTB (Figure 2c). The difference observed in these values is likely a consequence of steric hindrance by the large GST affinity tag partially blocking the binding site of *cyclo*-CIYYCV on BTB. We next probed the effect of *cyclo*-CIYYCV on protein stability by a thermal shift assay; the resulting data suggests that *cyclo*-CIYYCV increasingly stabilizes BTB with a dose-dependent increase in melting temperature observed for both GST-BTB (Supplemental Figure 1) and 3CysMut BTB (Figure 2d). We therefore sought to assess the role of potential disulfide bonds in the activity of our inhibitor.

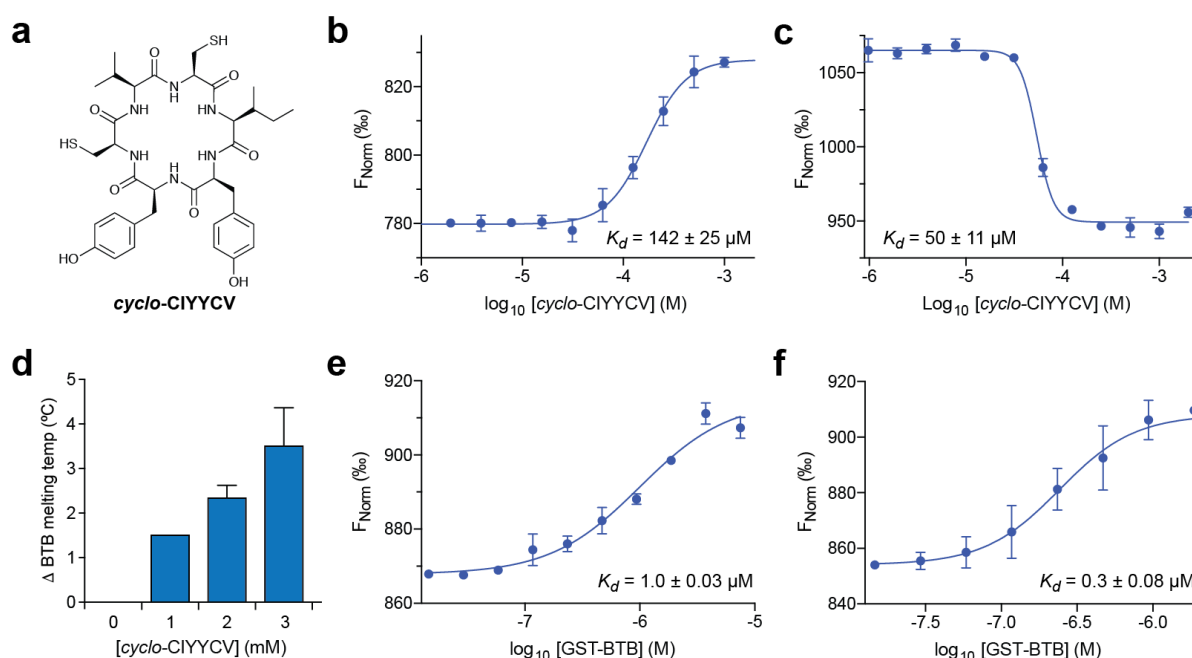


Figure 2: *in vitro* analysis of the binding of *cyclo*-CIYYCV to BTB. (a) Structure of *cyclo*-CIYYCV. (b) *cyclo*-CIYYCV binds to GST-BTB with a K_D of $142 \pm 25 \mu\text{M}$ by MST. (c) *cyclo*-CIYYCV binds to 3CysMut BTB with a K_D of $50 \pm 11 \mu\text{M}$ by MST. (d) *cyclo*-CIYYCV causes a dose dependent change in the melting temperature of 3cysMut BTB. (e) GST-BTB binds to fluorescently labelled SMRT2 peptide in the presence of 2.5 mM DTT, with a K_D of $1.0 \pm 0.03 \mu\text{M}$ by MST. (f) GST-BTB binds to fluorescently labelled SMRT2 peptide with a K_D of $0.3 \pm 0.08 \mu\text{M}$ by MST. All data presented as Mean \pm SEM, $n=3$.

Probing the requirement of disulfide bonds for *cyclo*-CIYYCV activity

To investigate this, the binding of our cyclic peptide inhibitor to BTB was assessed by MST in the presence of 2.5 mM DTT, which would be expected to disrupt disulfide bonds. We observed loss of all activity for *cyclo*-CIYYCV, against both the WT and 3CysMut protein (Supplemental Figure 2). To determine whether this observed loss of

activity was due to the DTT-mediated unfolding of BTB, we assessed the binding of the SMRT2 peptide to this protein in the presence and absence of DTT. It is well established that the SMRT2 corepressor peptide binds to the lateral groove of the BTB homodimer and cannot bind if the protein is misfolded, unfolded or in a monomeric state.[9] We synthesized a fluorescent derivative of the SMRT2 peptide (carboxyfluorescein-LVATVKEAGRSIWEIPR). This peptide was found to bind GST-BTB in the presence of DTT with a K_D of $1 \pm 0.03 \mu\text{M}$ (Figure 2e) by MST. Repeating this experiment in the absence of DTT, we observed a K_D of $0.3 \pm 0.08 \mu\text{M}$ for the same interaction (Figure 2f). This observed lack of effect from DTT on the binding of SMRT2 to GST-BTB indicates that DTT is unlikely to be affecting the tertiary and quaternary structure of BTB.

To ascertain whether the loss of *cyclo*-CIYYCV binding to BTB in the presence of DTT was dependent on intramolecular disulfide interactions between the two cysteines of the cyclic peptide, an Ellman's assay was performed, which may be used to determine the number of free cysteines in a molecule. We observed an absorbance approximately double that of a control containing one free cysteine residue (Supplemental Figure 3), indicating that in the absence of DTT and under assay conditions, both cysteine residues of *cyclo*-CIYYCV were in a reduced state. Together, this data indicates that the observed loss of activity associated with the presence of DTT is likely due to the disruption of disulfide interactions between the cyclic peptide and BTB. However, it is possible that despite the presence of DTT, an intramolecular disulfide bond persists in *cyclo*-CIYYCV and is required for its activity. One of the cysteines in such a species may still form a disulfide bond to BTB via disulfide shuffling.

Disruption of BTB homodimerization by *cyclo*-CIYYCV *in vitro*

We next used size exclusion chromatography (SEC) to assess the effect of *cyclo*-CIYYCV on the BTB dimer to monomer equilibrium. The 3CysMut protein was used in this experiment to avoid false negatives with BTB-GST arising from GST homodimerization. We observed a small shift in the 3CysMut BTB protein elution volume (from 2.18 mL to 2.22 mL) in the presence of our cyclic peptide inhibitor. This corresponded to a potential 12 kDa shift towards monomeric BTB (Figure 3a), consistent with data from the BTB RTHS (Figure 1c) that *cyclo*-CIYYCV disrupts the BTB homodimer. We further sought to verify the effect of our inhibitor on the dimerization status of BTB using an indirect assay; the SMRT2 corepressor peptide is known to bind to the lateral groove formed at the interface of the BTB homodimer, thus a BTB homodimerization inhibitor would be expected to reduce the apparent affinity of this interaction. We therefore used a competitive MST assay to measure the affinity of

SMRT2 for 3CysMut BTB in the presence and absence of *cyclo*-CIYYCV. We observed a K_D of $25 \pm 10 \mu\text{M}$ for SMRT2 binding to BTB in the absence of our cyclic peptide (Figure 3b), consistent with previously published data.[9, 13, 26] In line with our hypothesis, we observed a ~3-fold reduction in the affinity of this interaction in the presence of *cyclo*-CIYYCV (300 μM), with a K_D of $81 \pm 14 \mu\text{M}$ measured for SMRT2 binding to BTB in the presence of our cyclic peptide (Figure 3c).

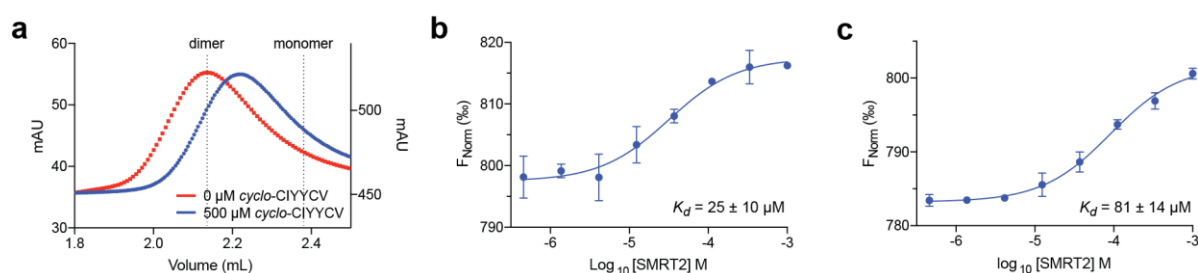


Figure 3: Assessing the ability of *cyclo*-CIYYCV to disrupt BTB dimerization. (a) Size exclusion chromatography of 3CysMut BTB incubated in the presence and absence of *cyclo*-CIYYCV, the data shows BTB shifts towards monomer in the presence of the inhibitor. (b) 3CysMut BTB binds to the SMRT2 peptide with a K_D of $25 \pm 10 \mu\text{M}$ by MST. (c) 3CysMut BTB binds to the SMRT2 peptide in the presence of *cyclo*-CIYYCV (300 μM) with a K_D of $81 \pm 14 \mu\text{M}$ by MST, a 3-fold reduction in affinity in the presence of the inhibitor.

Identifying the critical residues of *cyclo*-CIYYCV by alanine scanning

The residues of *cyclo*-CIYYCV that are critical for binding to BTB were identified by alanine scanning. We synthesized six derivatives of the parent cyclic peptide, each with a single amino acid replaced with alanine. The relative activity of each of these molecules (1 mM) was assessed by single-point MST; with a drop or loss of signal amplitude relative to the parent molecule, indicating reduced affinity or lack of binding. The data indicated that each of the two cysteines was critical for activity, with replacement of either with alanine resulting in loss of binding (Figure 4a). Surprisingly, only the second tyrosine was critical to activity of the cyclic peptide, whereas replacement of the first tyrosine only resulted in minor activity loss (Figure 4a). Replacement of either the isoleucine or valine with alanine also didn't affect binding to BTB (Figure 4a). Overall 3 of the 6 amino acids in *cyclo*-CIYYCV were found to be essential for its activity (Figure 4b). This data provides further evidence for disulphide bonding playing an important role in the activity of this molecule.

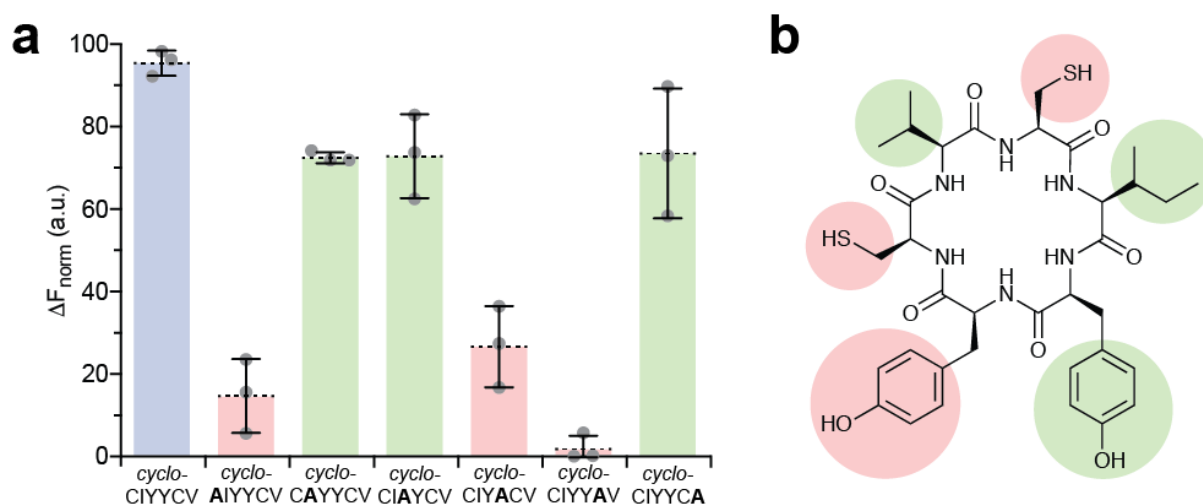


Figure 4: The critical residues of *cyclo*-CIYYCV. (a) Comparing the effect of 1mM of *cyclo*-CIYYCV with its 6 alanine-scanning analogues on the MST signal of fluorescently labelled BTB protein. Binding affinity of each molecule for BTB correlates to signal amplitude (i.e. low signal = weaker binding). (b) The structure of *cyclo*-CIYYCV with residues critical for its activity highlighted in red. Residues whose replacement with alanine did not have a large effect on binding to BTB are highlighted in green.

Conclusions

Taken together, the above data demonstrates that *cyclo*-CIYYCV binds to BTB and disrupts its homodimerization. The disparity between the apparent affinity of BTB monomers for each other, versus the binding constant of our cyclic peptide to BTB, suggests either a non-reversible mechanism of inhibition, or targeting of an allosteric site. We observed that DTT abrogates the activity of this molecule, and our data suggests that this may be due to the disruption of an intermolecular disulphide bond between the inhibitor and BTB. It is interesting to note that the reducing intracellular environment of the *E. coli* host used in our screen is likely to disfavour disulphide bond formation, yet *cyclo*-CIYYCV was selected in this screen and disrupts BTB homodimerization in the RTHS. Regardless of its mechanism of action, the moderate potency of the identified inhibitor limits its further characterization (both *in vitro* and in cells), and there is a need to improve the potency of our inhibitor. This may be achieved by improving non-covalent affinity via incorporation of non-natural amino acids, as we have previously demonstrated for other peptides.[19, 27, 28] Alanine-scanning of the cyclic peptide identified the two cysteines and the second tyrosine as critical to the activity of our BTB inhibitor. These residues, particularly the tyrosine may be substituted with non-natural analogues, while one or both cysteines may be replaced with a cysteine-trapping residue such as an acrylamide. Thus, this first in class inhibitor, identified from a genetically encoded SICLOPPS library provides a good foundation for

development of future BTB homodimerization inhibitors, which may serve as tools or lead molecules for the development of therapeutic agents targeted toward DLBCL.

Acknowledgements

The authors thank Dr. Nichole O'Connell for helpful discussions, and AstraZeneca and the Engineering and Physical Sciences Research Council for funding this work via a PhD studentship to E.L.O.

Supplementary Information

Supplementary Figures 1, 2 and 3; Supplementary Tables 1 and 2; and Material and Methods.

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